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Accumulation of Scoparone in Heat-Treated Lemon Fruit Inoculated with *Penicillium digitatum* Sacc.[1]

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ABSTRACT

Phytoalexin scoparone (6,7-dimethoxycoumarin) generally was not detected in noninoculated lemon fruit (*Citrus limon* [L.] Burm., cv Eureka) but accumulated in fruit after inoculation with *Penicillium digitatum* Sacc. A much greater increase in the amount of scoparone was found in fruit exhibiting an incompatible response to *Penicillium* after heat treatment at 36°C for 3 days. Heat treatment prevented development of decay in the inoculated fruit. The concentration of the compound after inoculation continued to increase during and after the heat treatment period, reaching 178 micrograms per gram fresh weight of the flavedo 6 days after the heat treatment. Changes in scoparone concentration in fruit were closely correlated with the changes in the antifungal activity of the fruit extract. A low concentration of the phytoalexin was detected in fruit injured mechanically. Scoparone also accumulated in the fruit following ultraviolet illumination; the concentration of the compound was dose-dependent. Median effective dose values of the inhibition of germ tube elongation and spore germination of *P. digitatum* were 29 and 46 micrograms per milliliter, respectively. Our findings suggest that the rapid increase in scoparone concentration plays an important role in the increased resistance of heat-treated lemon fruit to infection by *P. digitatum*.

Phytoalexins are frequently produced in the infected tissues of plants. It has been generally accepted that the phytoalexins play an important role in resistance against pathogens (15, 18, 19, 22). The presence of phytoalexins in citrus tissue has been reported also (1–5, 9–11, 16, 21, 23, 25).

Accumulation of antifungal compounds in citrus tissues was observed following inoculation with *Phytophthora citrophthora* Smith & Smith (23). Several coumarins, including xanthyletin (21) and seselin (25), accumulated in trunk and root tissue of citrus infected by *Phytophthora* spp. Ismail *et al.* (20) reported that the synthesis of umbelliferone (7-hydroxycoumarin) was greatly enhanced during healing of injured grapefruit. Afek and Sztejnberg (1, 2) isolated another coumarin, scoparone, (6,7-dimethoxycoumarin) from the bark of citrus following inoculation with *P. citrophthora*. This compound was induced in fruit, leaves, and twigs of Satsuma mandarin infected with *Diaporthe citri* (Faw.) Wolf and was not detected in the healthy tissue of the citrus (3–5). Scoparone was isolated also from orange peel infected with *Guignardia citricarpa* Kiely, the cause of black spot (16), and from peel of gamma-irradiated grapefruit (24), but it was not found in the nonirradiated fruit or in fruit not infected by the pathogen. Ben-Yehoshua *et al.* (11, 12) isolated several antifungal substances from pomelo fruit, some of which are coumarin derivatives. Recently, Ben-Yehoshua *et al.* (9) also detected scoparone in various citrus fruits following UV illumination. However, Baudoin and Eckert (7) did not find any significant accumulation of antifungal compounds in wounded tissues of lemons inoculated with *Geotrichum candidum* Link ex Pers.

Heat treatment of sealed citrus fruits at relatively high temperatures accelerated healing of fruit wounds and markedly reduced green mold decay caused by *Penicillium digitatum* Sacc. (10–12). Seal-packaging is essential for the success of the heat treatment by providing a water-saturated atmosphere and protecting the fruit from high temperature damage (8, 12).

The purpose of this work was to establish the role of phytoalexins in citrus fruit and to study their involvement in resistance of lemon fruit inoculated with *P. digitatum* in relation to heat treatment. It was found that the rapid accumulation of scoparone contributes greatly to the increased resistance of the heat-treated and inoculated fruit.

MATERIALS AND METHODS

Plant Materials

Mature, light-green lemons (*Citrus limon* [L.] Burm., cv Eureka) were obtained directly from orchards or packing houses in southern Israel before any postharvest treatment had been applied. Samples of fruit of uniform size and appearance, originating from one orchard, were subjected to different treatments at random. The lemons were washed with 70% ethanol and divided into as many groups as required for each experiment.

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Pathogen

P. digitatum isolated from infected fruit was cultured on potato dextrose agar (Difco) for 10 d at 24°C. Spores were washed and collected by filtration through eight layers of cheesecloth to remove most of the hyphal fragments, and the concentration of the spore suspension was adjusted to 10^6 mL^{-1} with a hemacytometer just before inoculation.

Inoculation

Fruits were inoculated by piercing the flavedo with a tool incorporating three 0.5-mm-diameter needles to a depth of 1.5 mm at four sites near the stem end of the fruit. The tool was immersed in a suspension of *P. digitatum* spores prior to the piercing operation. Each treatment consisted of 40 fruits with three replications.

The isolation of the phytoalexin was carried out on 600 fruits that were inoculated, as described above, at 20 sites on each fruit. One hundred fruits were mechanically injured in the same manner with the same tool as described above.

Seal-Packaging and Heat Treatment

Seal-packaging of lemon fruit was done with D-950 film (supplied by W.R. Grace, Cryovac Division, Passirana di Rho, Milan, Italy) immediately after inoculation. The inoculated and sealed fruits were maintained for 24 h at 17°C and 85% RH to allow the pathogen to start growing after inoculation. Inoculated and sealed fruits were heat-treated at 36°C for 3 d according to Ben-Yehoshua *et al.* (12). The inoculated and nonsealed fruit was maintained under water-saturated conditions achieved by holding fruit in a tray covered with a plastic film. The sealed fruit could be heat-treated at low humidity, because the microatmosphere of such fruit is normally water-saturated. After the heat treatment, the fruit was transferred to 17°C and 85% RH.

UV Illumination

Illumination of lemon fruit was carried out with various doses of UV light (254 nm) from a germicidal G15T8 lamp (supplied by Tana Co., Haela, Israel). The fruit was placed 25 cm from the UV source. After UV treatment, the fruit was kept at 17°C and 85% RH.

Preparation of Crude Extract

Flavedo tissue of the inoculated sites of lemon fruits (20 g) was excised with a scalpel and extracted for 1 d with petroleum ether at a ratio of 1:4 (w/v). The tissue was homogenized in an Osterizer Cyclotrol blender, and the homogenate was filtered *in vacuo* through Whatman No. 1 filter paper. The residue was rehomogenized with the same volume of petroleum ether and filtered *in vacuo*. This filtrate was dried over anhydrous $MgSO_4$ and concentrated *in vacuo* on a Buchi Rotavapor RE 120. The extract was collected with dichloromethane and dried under nitrogen gas. The dry sample was kept in a freezer (−15°C) in vials with nitrogen gas until use.

Chromatography of Crude Extract

Crude extracts (5 mg) were dissolved in dichloromethane (0.5 mL). Samples of these solutions (20 μL) were subjected to TLC on silica gel 60 F_{254}, 0.2 mm thick (Merck), with toluene:ethyl acetate (4:1, v/v) as the solvent. The developed plates were dried and exposed to UV light for detection of the fluorescent compounds.

Detection of Antifungal Activity from Crude Extracts

The crude extracts (4 mg each) were dissolved in ethanol (0.2 mL), after which 1.8 mL sterile water was added. A sample of this solution (40 μL) was put into a 35-mm-diameter dish (Sterilin Ltd., Feltham, UK) that has four wells (11 mm diameter). Each well had 40 μL of *P. digitatum* spore suspension (2.5×10^4 spores mL^{-1}) containing 1% fresh orange juice and 1% sucrose at pH 3.7. The prepared dish was placed in a humid tray covered with plastic film and incubated at 17°C for 20 h. The assay was repeated in triplicate.

The percentage of germination and germ tube elongation of *P. digitatum* spores were measured to evaluate the antifungal activity. The method was standardized with synthetic scoparone (Merignac).

Isolation of the Antifungal Substances Induced by Combined Treatments

The crude extract of the combined treatment was dissolved in dichloromethane, placed on TLC plates, and developed with toluene:ethyl acetate (4:1, v/v). The developed plates were exposed to UV light (366 nm). Individual bands were collected and extracted with dichloromethane. The extract was concentrated *in vacuo,* collected with dichloromethane, and rechromatographed with a more polar solvent, toluene:ethyl acetate (1:1, v/v). Bands were recollected, extracted, and concentrated *in vacuo*. The extract was dried under nitrogen gas and used in the bioassay for antifungal activity and for quantitative determination of the compound.

Detection of Antifungal Activity on TLC Plates

Antifungal activity of the TLC bands was bioassayed directly on the plates with *Cladosporium cladosporioides* G.A. De Vries as the test organism (14).

Dilution series of the band extracts, starting from 5 mg mL^{-1}, were prepared with dichloromethane. Samples (20 μL) of the dilution series were placed onto a TLC plate, and the plate was developed with toluene:ethyl acetate (1:1, v/v). The plate was air-dried overnight at room temperature and sprayed with a *C. cladosporioides* spore suspension (10^6 spores mL^{-1}) in Czapek-Dox medium. The sprayed plate was placed in a humid tray covered with plastic film and incubated at 24°C for 2 to 3 d. Antifungal activity was indicated by the absence of mycelium around the spot of the compound on the plate.

HPLC, UV, IR, ^{1}H-NMR, and ^{13}C-NMR of Active Fraction

The active fraction was purified by HPLC on a Varian 5000 equipped with variable wavelength UV-50 detector and

a C-18 reverse phase column (250 × 4 mm, Merck). The elution solvent mixture was methanol:water (4:1, v/v), and the flow-rate was held constant at 0.5 mL min^{-1} for 12 min. Peaks were detected with a fixed wavelength of 335 nm. The absorbance spectrum of the compound obtained by HPLC was taken with a Uvikon spectrophotometer with methanol as solvent. The structure of the tested compound was confirmed by its IR spectrum in KBr pellet with a Perkin-Elmer model 177 spectrophotometer, as well as by ^{1}H-NMR spectrum at 360 MHz and ^{13}C-NMR spectrum at 90 MHz in $CDCl_3$ on a Bruker WM-360 spectrometer.

Quantitative Determination of Scoparone Extracted from Lemon

Quantitative analysis was performed on a SPF-125 spectrophotofluorometer (Aminco). The analysis was carried out with samples separated from the crude extracts on a TLC plate as described above. The scoparone spot was eluted with 5 mL of methanol and centrifuged. Standardization of the analysis was done with an authentic scoparone sample. The relative fluorescence intensities at excitation of 300 nm and at emission of 430 nm were in positive linear correlation with the concentration of the compound ($y = 1.39 + 21.21x$, $r^2 = 0.99$). The quantitative analysis of scoparone extracted from lemon was repeated in triplicate.

RESULTS

Effect of Heat Treatment on *Penicillium* Decay

As shown previously (12), heat treatment at 36°C for 3 d prevented development of *Penicillium* decay in inoculated and sealed fruits for more than 2 months of storage at 17°C. Nonheat-treated fruits rotted in 3 to 7 d after inoculation.

Effect of Heat Treatment on the Composition of Fluorescent Compounds

Heat treatment alone did not affect the composition of fluorescent substances in fruit. The chromatogram of crude extract from noninoculated fruit 6 d after heat treatment showed the same composition as that from control fruit. When compared with control, the extract from inoculated and subsequently heat-treated fruit showed a single new band at R_F 0.25, which emitted bright blue fluorescence.

Effect of Heat Treatment Alone and in Combination with Inoculation on the Antifungal Activity of the Crude Extract

All tested extracts inhibited germination of *P. digitatum* spores and germ tube elongation, showing the presence of antifungal substances. The crude extract from the fruit just after harvest inhibited germ tube elongation and spore germination 45, and 32%, respectively (Fig. 1). The low initial antifungal activity of the extract from nontreated fruit declined continuously during the storage period, and, after 70 d of storage, this extract inhibited germination and germ tube elongation of *P. digitatum* spores 11 and 15%, respectively. The antifungal activity of crude extract did not increase after heat treatment of noninoculated fruit, but the heat-treated fruit maintained its initial antifungal activity from harvest time during 70 d of storage. The antifungal activity of the crude extract prepared from heat-treated lemon 10 d after inoculation was two- to threefold higher than that of the crude extract from the fruit just after harvest (Fig. 1). Later, the antifungal activity declined, but the extract from fruit stored for 40 d after inoculation still had higher antifungal activity than the crude extract from the fruit just after harvest. The level of inhibition exhibited by the extract from the fruit stored for 70 d after inoculation was lower than that of the crude extract from the fruit just after harvest.

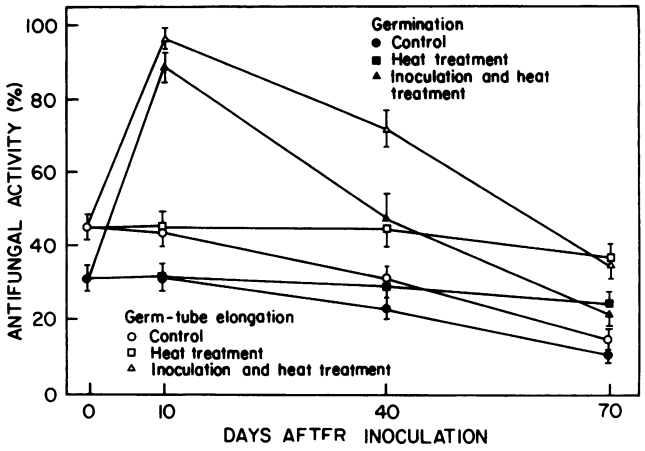

Figure 1. Effect of heat treatment on the antifungal activity of the crude extracts from inoculated and noninoculated lemon fruits. Fruits were inoculated with a spore suspension (10^6 spores mL^{-1} of *P. digitatum*. Fruits were sealed immediately after inoculation. The heat treatment was applied for 72 h at 36°C 24 h after inoculation. Except during the heat treatment period, all the fruits were kept at 17°C and 85% RH. The antifungal activity of the crude extracts was expressed as percentage of inhibition of germination of *P. digitatum* spores and germ tube elongation as compared with that of the control treatment containing 0.5% sucrose, 0.5% orange juice, and 5% ethanol. The assay was repeated in triplicate.

Detection of Antifungal Activity Induced by Combined Treatments

After inoculation and heat treatment of the fruit, dilution series of the induced fluorescent compound were examined for antifungal activity toward the fungus *C. cladosporioides* by TLC-bioassay. The areas of fungal growth inhibition at R_F 0.5 on the TLC plate were larger at the higher concentrations of the compound. This antifungal activity was detected even at 6.3 μg of the compound on the TLC plate.

Identification of the Induced Compound

The induced compound was further purified by HPLC. The results are shown in Figure 2. The peak obtained was collected and analyzed by IR, ^{1}H-NMR, and ^{13}C-NMR. The results from HPLC and TLC (two carriers) analysis, and the UV, fluorescence, IR, ^{1}H-NMR, and ^{13}C-NMR spectra, verified the identification of the compound as 6,7-dimethoxycou-

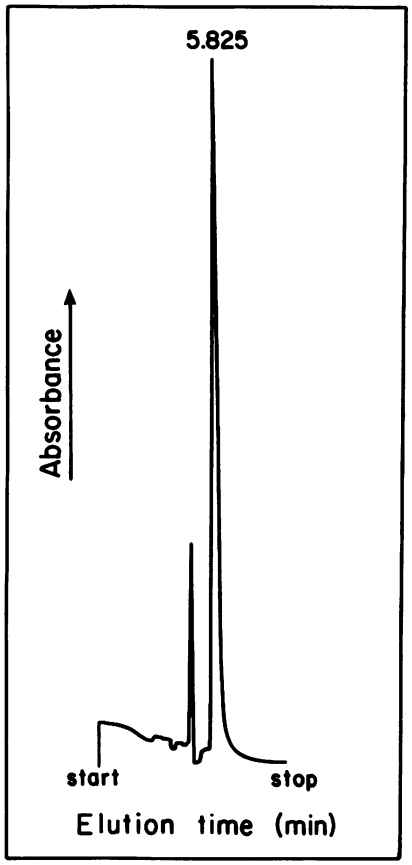

Figure 2. HPLC analysis of the compound induced by a combined inoculation with *P. digitatum* and heat treatment in lemon fruit. The peak eluting at 5.8 min was subsequently identified as scoparone. This analysis was done on a C-18 reverse phase column (250 × 4 mm) eluted with 80% methanol in water (0.5 mL min^{-1}). A UV detector was fixed at a wavelength of 335 nm.

marin (scoparone) compared with results of standard scoparone (2, 5, 16, 24).

Estimation of Antifungal Activity of Scoparone

The antifungal activity of scoparone was determined by bioassay with *P. digitatum* spores. The percentage of germination and germ tube elongation were negatively correlated with the concentration of scoparone (Fig. 3). At 500 $\mu g\ mL^{-1}$, the compound completely inhibited germination of the spores and germ tube elongation. Spore germination was less sensitive to the compound than germ tube elongation. The median effective dose values were 29 and 46 $\mu g\ mL^{-1}$ for the inhibition of germ tube elongation and germination of *P. digitatum* spores, respectively.

Accumulation of Scoparone in *Penicillium*-Inoculated Lemon

Scoparone accumulated in lemon fruit after inoculation with *P. digitatum* but was not detected in noninoculated fruit. However, in one out of five experiments, we found trace amounts of scoparone, approximately 1 to 2 $\mu g\ g^{-1}$ fresh weight of flavedo. Figure 4 shows that scoparone appeared after inoculation in both heat-treated and nonheat-treated fruit, but its concentration was much higher and increased more rapidly in the heat-treated fruit. The concentration of the compound increased to 109 $\mu g\ g^{-1}$ fresh weight in the fruit heat-treated for 72 h and continued to increase up to 178 $\mu g\ g^{-1}$ fresh weight in the fruit by 6 d after the heat treatment. After reaching that peak, scoparone concentration declined rapidly to less than 6 $\mu g\ g^{-1}$ fresh weight in the fruit 70 d after inoculation. A low concentration of scoparone accumulated in the inoculated fruit that was not heat-treated. Because these nonheat-treated fruits rotted several days after inoculation, no data on scoparone level could be collected beyond this period (Fig. 4).

Scoparone was detected by TLC only in the extract from the inoculated site of the fruit. In the extract from the opposite side of the fruit, or even of flavedo tissue 1 cm away of the inoculated site, this compound was not detected.

Accumulation of Scoparone in Mechanically Injured Lemon

Low concentrations of scoparone were found in flavedo after injuring both heat-treated and nonheat-treated fruit. After 10 d of storage, 12 and 7 $\mu g\ g^{-1}$ fresh weight of scoparone accumulated in the nonheat-treated and heat-treated fruit, respectively.

Scoparone Induction in Lemon by UV Illumination

Substantial amounts of scoparone accumulated in lemon fruit illuminated with UV light. Concentrations of scoparone were 27, 77, and 120 $\mu g\ g^{-1}$ fresh weight in fruit illuminated with 1.5, 3.0, and 4.5 × 10^4 erg mm^{-2} after 7 d incubation, respectively. Later, the scoparone concentration declined (Fig. 5).

DISCUSSION

The present study demonstrates the induction of scoparone synthesis in flavedo tissue of lemon after inoculation with *P.*

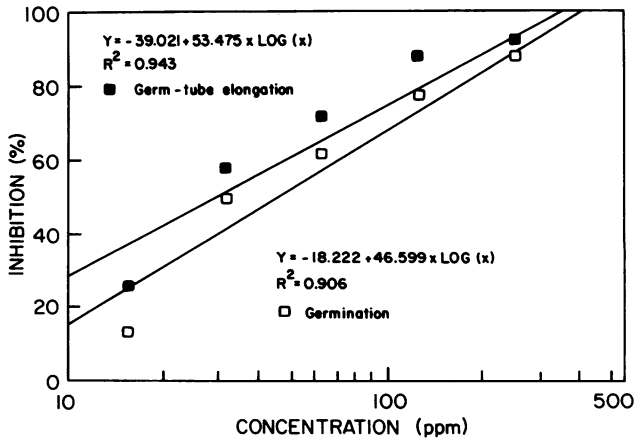

Figure 3. Inhibitory activity of scoparone on spore germination and germ tube elongation of *P. digitatum*.

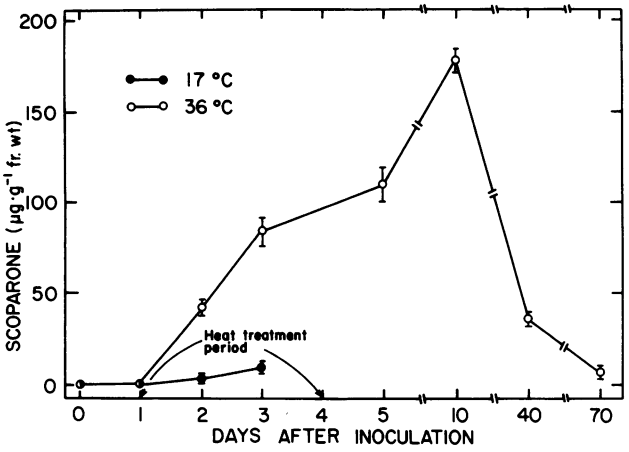

Figure 4. Effect of heat treatment on accumulation of scoparone in lemon flavedo inoculated with *P. digitatum*. The inoculation, sealpackaging, heat treatment, and fruit storage were as described in Figure 1. The quantitative assay was repeated in triplicate. Temperature indicated is only for the period of heat treatment. During the rest of the period both treatments were at 17°C and 85% RH.

digitatum. Furthermore, accumulation of this compound increased rapidly following heat treatment of inoculated fruit. This level of scoparone accumulating at the inoculated site was sufficient to inhibit the growth of the fungus in the fruit. After reaching a peak on the sixth day after heat treatment, the concentration of the compound declined. Such a pattern of accumulation and degradation is typical of phytoalexins in plant tissues (6). Heat treatment alone did not result in the accumulation of scoparone in lemons.

Only low concentration of scoparone accumulated as a result of mechanical injury, demonstrating the major role of pathogen in elicitation of the defense response. Afek and Sztejnberg (2) reported that mechanical wounding did not induce the scoparone accumulation in citrus bark. However, Arimoto and Homma (3) showed the induction of scoparone in citrus leaves by mechanical injury.

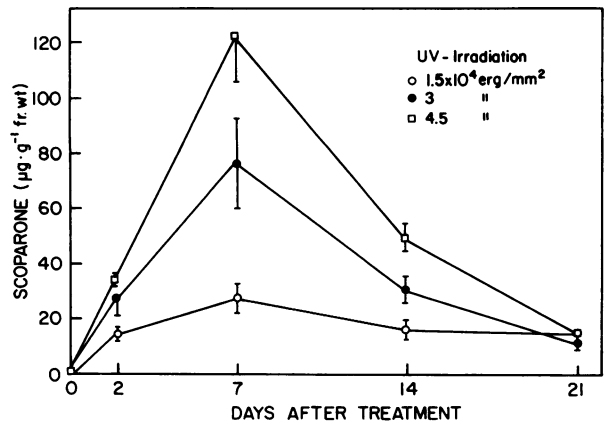

Figure 5. Effect of UV dose on accumulation of scoparone in lemon fruit. Fruits were incubated at 17°C and 85% RH after UV treatment. The quantitative assay was repeated in triplicate.

Scoparone accumulated at the inoculation sites of lemon fruit but was not detected on the side opposite the inoculation site or even 1 cm away from the inoculation site. This observation agrees with the report (19) that phytoalexin biosynthesis is confined to the infected cells and their immediate vicinity in most plant species.

UV illumination was also an effective elicitor of phytoalexin. This is in agreement with an earlier report (17). It appears then that, unlike heat, mechanical injury and UV illumination induced production of scoparone even without *Penicillium* inoculation in lemons and other citrus fruits (9).

Changes in scoparone concentration in fruit were closely correlated with changes observed in antifungal activity of extracts from this fruit. Antifungal activity of extracts from inoculated fruit increased following heat treatment. This could be the result of rapid accumulation of scoparone at the inoculation site. Antifungal activity did not increase following heat treatment alone. However, heat treatment markedly prevented the decline of antifungal activity in fruit during storage. The crude extract at harvest also inhibited germination and germ tube elongation of *P. digitatum* spores, resulting from the presence of preformed antifungal substances other than scoparone in fruit (4, 10, 11).

Baudoin and Eckert (7) did not find any phytoalexin or antifungal activity in lemons, probably because (among other possibilities), unless heat is applied, decay develops rapidly and, hence, little scoparone accumulates.

The effect of temperature on scoparone production has been studied by Arimoto and Homma (3), who found that the largest quantity of this compound was produced in the melanose spots or scars at 25°C, with progressively lower levels at lower temperatures until no scoparone was detected at 5 or 10°C. Afek and Sztejnberg (1) reported that the concentration of scoparone in the bark of rough lemon was sevenfold higher at 28 than at 20°C, in parallel with the increase of resistance to *P. citrophthora*.

To the best of our knowledge, this is the first report that demonstrates phytoalexin accumulation in lemon fruit. Scoparone was absent in noninfected lemon flavedo and appeared only after *Penicillium* inoculation. It should be noted, however, that on rare occasions scoparone at very low levels was detected in healthy fruit that was not stressed. The concentration of scoparone evoked by heat treatment was adequate to inhibit the development of decay. These points support previous conclusions (1–5) that scoparone is a phytoalexin. The present investigation suggests that the rapid increase in scoparone concentration plays an important role in the increased resistance of heat-treated lemon fruit to infection by *P. digitatum*.

The lignification process is commonly accepted as another mechanism of resistance of citrus fruit to pathogens (7, 12, 13). The relative contributions of lignification and scoparone induction to the resistance of citrus fruit, in general, and to the efficacy of the heat treatment, have not been investigated.

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